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**Advanced analytical techniques for studying the morphology and
chemistry of Proterozoic microfossils**

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Abstract: This contribution outlines the suite of **advanced** multiscalar techniques in the
toolkit of the modern Proterozoic palaeobiologist. These include non-intrusive and non-
destructive optical, laser and X-ray based techniques, plus more destructive ion beam and
electron beam based methods. Together, these provide morphological, mineralogical and
biochemical data at flexible spatial scales from that of an individual atom up to that of the
largest of Proterozoic microfossils. Here we provide a description of each technique,
followed by a case study from the exceptionally preserved Torridonian biota of Northwest
Scotland. This microfossil assemblage was first recognized over a century ago, but its great

diversity and evolutionary importance has only recently come to light, due in no small part to the research efforts of Martin Brasier.

Running Title: Proterozoic microfossil techniques

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ION BEAM–LASER RAMAN–CONFOCAL LASER SCANNING MICROSCOPY–
SECONDARY ION MASS SPECTROMETRY–3D VISUALISATION

INTRODUCTION

Modern palaeobiology primarily exists to discover, describe, and decode the ancient biosphere, and understand the course of global evolutionary change. Stemming from its roots in Victorian natural history, palaeobiology has made good use of technological advances to shed light on new discoveries (see Sutton *et al.* 2014, Wacey, 2014 and references therein), and to reveal previously unimagined details in historical material (Brasier *et al.* 2015). As with any modern field of science, palaeobiological research must continually look forwards to the next potential discovery, utilizing all available tools and techniques.

Historically, major discoveries have predominantly dated from the Phanerozoic, owing to the relatively well-preserved and easily recovered fossils of the macroscopic organisms alive during this time. In the search for life’s origins and early record, attention has inevitably turned to the more poorly understood Proterozoic and Archaean fossil records. The evolutionary history of these expanses of time is much less well established, as a result of the

57 shortage of exposed rock of appropriate age, relative paucity of fossil material, and
58 limitations in extracting relevant information. Fossils from these times are typically
59 microscopic, enigmatic, and poorly preserved, although a number of exceptionally preserved
60 deposits have come to characterize the Proterozoic fossil record (e.g. Torridonian biota,
61 Strother *et al.* 2011; Doushantuo biota, Yin & Li 1978). In both ‘traditional’ and
62 ‘exceptional’ preservation cases, our understanding remains limited by the observational and
63 analytical techniques used to characterize these important specimens.

64

65 The approaches traditionally used to study early fossil material are essentially borrowed and
66 adapted from methods used in the study of Palaeozoic fossils, and are best suited to hard-
67 bodied macroscopic fossils or compressed organic material extracted by acid maceration.
68 However, as our understanding of Precambrian environments is fundamentally improving, it
69 has become clear that entirely different preservational styles are possible, some of which
70 require novel analytical approaches. Although many Proterozoic carbonaceous fossils can
71 still be found compressed within shales (Javaux *et al.* 2004; Agic *et al.* 2015), and extracted
72 for study by palynological acid maceration techniques, microfossil material can be hosted in
73 a variety of other media, including chert (e.g. Barghoorn & Tyler 1965), pyrite (e.g.
74 Rasmussen 2000), authigenic aluminosilicates (e.g. Wacey *et al.* 2014), and cryptocrystalline
75 phosphate (e.g. Strother *et al.* 2011). These alternative preservational styles originate from
76 the biogeochemical conditions that prevailed in specific environments or across specific
77 periods of time, and are able to exceptionally preserve microfossils of a wide range of
78 affinities in their original spatial context, often in three dimensions, and reflecting a broad
79 spectrum of taphonomic decay. In these cellular lagerstätte, challenges are posed by the small
80 scale, enigmatic nature, and relative scarcity of Proterozoic fossils, as well as by their
81 complex taphonomic and metamorphic histories. Thus, a thorough understanding of

82 Proterozoic and Archaean life necessarily calls for state-of-the-art high-spatial-resolution,
83 and holistic imaging and analysis techniques.

84

85 An increasing number of researchers are now making use of such techniques to study both
86 Proterozoic and Archaean material, revealing unprecedented levels of detail and allowing the
87 reconstruction of the complex Precambrian biosphere. It is still common, however, for these
88 different approaches to be attempted separately, often by different individual research groups,
89 which can partially preclude the synthesis of information and an overall understanding of
90 local, regional, or even global palaeoecologies. Here we present a holistic methodology for
91 studying Proterozoic fossil deposits, with a consideration of their unique preservational styles
92 and histories. A set of complementary microanalysis techniques have already been presented
93 with respect to Archaean material (Wacey 2014). However, with the expansion of the
94 biosphere (Knoll 1994), the evolution of Eukaryotic cells (Knoll *et al.* 2006), and the advent
95 of various metabolic pathways and trophic tiering (Knoll 2015), the Proterozoic fossil record
96 is more complex and - owing to its younger age (approximately 2500–540 Ma) - arguably
97 better preserved. Thus, a greater potential wealth of information might be gleaned from such
98 deposits, necessitating their study on a variety of spatial scales, as well as assessing both
99 morphology and chemistry.

100

101 The following sections detail, in a logical order for practical investigation, multiple
102 approaches for examining a Proterozoic microfossil assemblage, including: ‘traditional’ field
103 study and optical microscopy; X-ray based techniques including X-ray tomography and X-
104 ray spectroscopy; laser-based techniques including Raman spectroscopy and confocal laser
105 scanning microscopy; infrared spectroscopy; electron based techniques including scanning
106 electron microscopy and transmission electron microscopy; and ion based techniques

including focused ion beam milling and secondary ion mass spectrometry. The combination of several of these techniques when investigating a single fossil deposit provides the best opportunity to fully reveal the palaeocology of the Proterozoic biosphere. An example of their application to the microfossiliferous rocks of the 1200-1000 Ma Torridonian Supergroup of northwest Scotland is subsequently presented as a demonstrative case study.

STANDARD PALAEOBIOLOGICAL TECHNIQUES

Field Study and Optical Microscopy

A critical starting point for any palaeobiological investigation remains a comprehensive field study and the preparation of candidate material for optical microscopy. As a preliminary investigation, this can provide important palaeoenvironmental context, and enable the quantification of the richness, morphology, and spatial distribution of fossils, plus the depositional setting and taphonomic history of the fossil deposit.

Initially, a detailed sedimentological and stratigraphic study should be made of the fossiliferous rocks, and those associated with them, to permit accurate palaeoenvironmental, metamorphic, and tectonic interpretation. Such a study will provide regional, local, and fine scale information pertaining to the location, type, and energy of the environment of deposition, as well as any subsequent chemical or structural changes that may have taken place since lithification. Fine scale field observations will also permit the identification of candidate fossiliferous material. This may be related to specific preservational mineralogies, such as cherts (e.g. ~1900 Ma Gunflint Formation; Barghoorn & Tyler 1965) or phosphates (e.g. ~600 Ma Doushantuo Formation; She *et al.* 2013), or be found in association with macroscopic fossil structures including **siliceous and phosphatic** stromatolites (e.g. ~1900 Ma Belcher Supergroup; Hofmann 1976) and microbially induced sedimentary structures (e.g.

~1000 Ma Diabaig Formation; Callow *et al.* 2011). Collection and documentation of candidate material should be methodical, and include GPS localities, orientation data and specific relationships to larger-scale structures.

From the collected samples, polished, uncovered (which are more useful than covered for subsequent techniques) petrographic thin sections can be prepared for analysis using optical microscopy. Ideally, thin sections should be prepared both perpendicular and parallel to the bedding direction in order to capture the full spatial distribution of microscopic fossils. While sections 30 μm thick are required for mineral identification using cross-polarized light, the detection of fossil material may be facilitated by the use of sections up to $\sim 150 \mu\text{m}$ -thick, provided the encasing medium is sufficiently light-coloured and free of dark impurities. This increases the chances of capturing entire cellular material and in-situ relationships between different fossil taxa.

The primary purpose of optical microscopy is to locate and identify fossil material, and to document its spatial distribution and relationship with non-biological minerals. For the majority of Proterozoic carbonaceous fossil deposits, examination and imaging at all magnifications up to 1000x is needed to provide a complete context. This can allow the observation of fine structural details up to $\sim 0.2 \mu\text{m}$ across, but note at highest resolution oil immersion is required to increase clarity, which may be detrimental to some subsequent techniques. The position of fossil material can be identified and recorded for future reference using standard graticules, When fossil material is preserved with some degree of three-dimensionality, focusing through the thickness of the slide can reveal its shape, organization, and extent. A range of different photomicrography suites are now available for capturing images of such samples (e.g., *Synchroscopy Auto-Montage* as demonstrated by Brasier *et al.*

2005). Many packages contain algorithms for stacking focused images from different depths within a section to produce a single, focused image, or for stitching together images of adjacent fields of view to produce a high-resolution 'map' of a thin section.

Using a variety of optical micrographic tools, preliminary identification and quantification of fossil material may be carried out, larger-scale spatial relationships determined, and candidate fossils selected for further analysis. This work is vital for the initial study of a fossil deposit but the intrinsic limitations of this approach preclude its use for further, finer-scale analyses. Furthermore, certain media may be unsuitable for investigation by optical microscopy. Dark coloured material, or enclosing media with many impurities, for example, may mask fossil details and reduce their visibility, especially through thick sections. Larger microfossils may be cross cut by the sectioning process, limiting interpretation. Another limitation is that the identification of chemical constituents of samples is limited to that which can be determined by standard petrographic methods, and may not be sufficient for fine-grained or finely-crystalline material. Since carbonaceous fossils are frequently dark-coloured, optical analyses will also only be able to resolve their surface shape and structure, with the fossils themselves masking any underlying ultrastructure or interior features. Thus, for a better understanding of both fossil material and its preservational medium, more versatile high-spatial-resolution techniques are required.

Non-destructive moderate to high spatial resolution techniques

Non-destructive techniques are here classified as those which can be applied to a standard geological thin section, rock chip or rock hand sample with minimal further sample preparation and do not consume or alter the specimen of interest during the analysis. Hence,

they can be applied to both type specimens (including holotypes on loan from museums) and can be utilized as a precursor to more destructive techniques on newly discovered material.

X-ray Computed Tomography (X-ray CT)

X-ray CT maps the X-ray attenuation within a rotating sample. Data is captured as a series of projections that can then be reconstructed as two-dimensional slices and then three-dimensional visualisations (see [Kak & Slaney 2001](#); Cnudde & Boone 2013 for overviews). X-ray attenuation is dictated by factors such as elemental composition and density, hence X-ray CT can often detect variations in the style of fossil preservation and mineralization, as well as building up 3D models of entire specimens ([Conroy & Vannier 1984](#); [Haubitz et al. 1988](#); [Sutton et al. 2001](#)). The high resolution form of X-ray CT used for fossils is known as X-ray microtomography (μ CT), which has been utilized in palaeobiology for almost two decades ([Rowe et al. 2001](#); [Sutton 2008](#)). It is now routinely applied to Phanerozoic vertebrate and invertebrate fossils, ranging for example from echinoderms ([Rahman and Zamora, 2009](#)) to dinosaurs ([Brasier et al. 2016](#), this volume), and from plants ([Spencer et al. 2013](#)) to arthropods ([Garwood & Sutton 2010](#)). The study of microfossils using CT has become viable in recent years, with the use of synchrotron-based systems where more intense, monochromatic X-rays, can allow improved contrast and greater spatial resolution ([Donoghue et al. 2006](#); [Huldtgren et al. 2011](#)). Recent years have also seen improvements in the spatial resolution of lab-based ‘micro-CT’ and ‘nano-CT’ systems where sub- μ m resolutions are now possible ([Hagadorn et al. 2006](#); [Schiffbauer et al. 2012](#); [Sutton et al. 2014](#)).

Despite these technological advances, configuring the correct **instrumental** parameters for μ CT scanning a given microfossil specimen is challenging, and some specimens will not be

suited to μ CT due to lack of X-ray attenuation contrast between specimen and matrix **and/or**
presence of X-ray opaque minerals. In general, μ CT is applied to small rock chips. It is not
suited to geological thin sections because of their highly anisotropic nature, although thin
sections can be cut down to a more isotropic shape if permitted by the owner, or fossils
liberated using a micro-corer. Elsewhere in this volume, Hickman-Lewis *et al.* (2016)
provide several case studies of the μ CT scanning of Precambrian microfossil bearing rocks
using two lab-based CT scanners with spatial resolutions (minimum voxel sizes) of about
5 μ m and 0.5 μ m respectively. They show that μ CT can be a valuable tool to decode the 3D
petrographic context of such biological material, for example, by highlighting potential
organic grains and laminations, fractures within the matrix, assemblages of detrital heavy
minerals, and replacement of silica by carbonate rhombs (which are known to reduce the
quality of microfossil preservation). Detecting individual microfossils using lab-based CT
remains challenging unless the preservation window is particularly favourable (e.g. pyritised
microfossils in a silica matrix, see Hickman-Lewis *et al.* 2016, this volume). The use of a
synchrotron-based CT (or lab-based nano-CT) system can improve matters **by providing**
more intense X-rays and improved spatial resolution, but this requires more specialist sample
preparation (**e.g., micro-coring**) to obtain sub-mm pieces of fossiliferous rock, meaning that it
can no longer realistically be classified as a non-destructive technique, and could seldom be
applied to holotype material.

X-ray Spectroscopy

A logical extension to examining the morphology of microfossils using X-ray
microtomography is to investigate their chemistry using X-ray spectroscopy. A range of X-
ray techniques are available to characterize fossiliferous rocks, mostly performed on a
synchrotron beamline (see Templeton & Knowles 2009; **Fenter *et al.* 2002** for overviews),

and utilising both hard X-rays (more penetrating with wavelengths of 1-20 angstroms and photon energies over about 5-10 keV) and soft X-rays (less penetrating with wavelengths of 20-200 angstroms and photon energies below about 5 keV). X-ray fluorescence (XRF) mapping provides semi-quantitative element-specific maps over flexible spatial scales (μm to mm; e.g. Edwards *et al.* 2014). Near edge X-ray absorption fine structure (NEXAFS) and X-ray absorption near edge structure (XANES) are spectroscopic techniques, using soft (low energy) and hard (high energy) X-rays respectively, that excite core electrons in an element (Templeton & Knowles 2009). The resulting spectra provide information on both the coordination chemistry and valence of the element of interest. Scanning Transmission X-ray microscopy (STXM) uses soft X-rays to obtain both spectral data and images of this spectral data (e.g., maps of the spatial distribution of specific elements, valence states, or functional groups) at the nm-scale, created by rastering samples through an X-ray beam at stepwise-increasing incident X-ray energies to cover the absorption edges of the elements of interest (e.g., Lawrence *et al.* 2003). Although these types of analyses do not destroy the specimen, specialist sample preparation (e.g., micro-cored rock chips; doubly polished thin sections no more than about 100 μm thick) means that permission for holotype specimens to be analysed in this way is unlikely to be granted. Beam damage can also affect subsequent chemical analyses.

In terms of Proterozoic microfossils, much of the interest in X-ray spectroscopy surrounds the chemical bonding of carbon. The energy resolution of NEXAFS/XANES is excellent ($\sim 0.1\text{eV}$) so closely spaced peaks can be resolved. Hence, carbon bound, for example, in aromatic groups, aliphatic groups, ketones, peptides, carbonyls, carboxyls and carbonate can all be distinguished from one another (Bernard *et al.* 2007). Such spectra may help characterise cellular versus extracellular organic components, while interfering signals from

carbonate minerals can be subtracted. De Gregorio *et al.* (2009) applied this methodology to powders of organic material from the 1878 Ma Gunflint Formation, showing that polyaromatic carbon, carboxyl and phenol groups had all been preserved in this ancient kerogen. Similarly, the bonding characteristics of other elements common in organic material (e.g., S, N, P, O) may help to determine whether they are present as organic or inorganic forms in ancient fossiliferous rocks. For example, Lemelle *et al.* (2008) used XRF to quantify the amounts of sulfur within the cell walls of coccoid microfossils from the ~750 Ma Draken Formation, Svalbard, before using XANES to determine sulfur speciation. They showed that the sulfur was a reduced organic form most likely belonging to a thiophene-like compound.

Confocal Laser Scanning Microscopy (CLSM)

CLSM provides high-spatial-resolution morphological data (<100 nm is possible) permitting the visualisation of microfossils in three dimensions (see Sutton *et al.* 2014; Halbhuber & Konig 2003 for overviews). Under ideal conditions data collection from standard polished or unpolished geological thin sections is rapid and CLSM is able to resolve tiny morphological features that may be unclear or hidden when viewed under light microscopy, as well as giving a true 3D perspective to the distribution of microfossils (Schopf *et al.* 2006; Cavalazzi *et al.* 2011). However, natural samples are rarely ideal for the application of this technique. CLSM relies on the fact that organic material auto-fluoresces when excited by a laser of a specific wavelength. The system can accurately focus and scan at different depths within a microfossil specimen, and exclude the fluorescence from outside the plane of focus; 3D images are then built up combining data acquired from successive planes of focus (see Amos & White 2003). Hence, anything that interferes with the transmission or detection of this signal severely degrades the quality of the final images obtained. For example, specimens situated a long way below the surface of a thin section, or having thick opaque walls will not

provide sharp CLSM images. Similarly a specimen surrounded by plentiful fluorescing organic detritus, or embedded in a mineral that internally reflects the fluorescence signal, may be problematic. The maturity of the organic material also affects the quality of the data, with the auto-fluorescence signal dissipating as the organic material becomes more geochemically mature and loses more of its heteroatoms (i.e. evolves towards the structure of graphite). Hence, CLSM is of greatest use when applied to thin-walled organic microfossils, preserved in silica (and to a lesser extent phosphate), housed in rocks of low-metamorphic grade. In these cases significant insights into the three-dimensional morphology and taphonomic preservation of Proterozoic microfossils may be obtained. For example in the Neoproterozoic Buxa Formation, CLSM was able to demonstrate the three dimensional organisation of groups of filamentous microfossils (Schopf *et al.* 2008). In the 850 Ma Bitter Springs Formation and the 650 Ma Chichkan Formation notches, tears, grooves and surface ornamentation were all detected in microfossils using CLSM (Schopf *et al.* 2006), while in the ~580 Ma Doushantuo Formation CLSM revealed parts of fibrous tissues and cell walls within fossil alga that were not visible by other means (Chi *et al.* 2006).

Laser Raman Microspectroscopy and Imagery

Raman is a versatile, non-intrusive and non-destructive *in situ* technique. It can be used to identify the mineralogy of microfossils and their host rocks, and is particularly sensitive to the molecular structure and geochemical maturity of carbonaceous phases such as kerogen - the prime constituent of organic walled microfossils (see Beyssac *et al.* 2002 and Fries & Steele 2011 for details). In addition, when utilized in confocal imaging mode, Raman can provide 2D and 3D chemical and structural maps of microfossils at moderate spatial resolution (potentially < 1 µm). Raman can be applied to rock chips and standard uncovered geological thin sections. Data is acquired via laser excitation of the chemical bonds within the

sample. This excitation produces characteristic spectra depending on the minerals and compounds present, and maps can be constructed of the spatial distribution of various spectral parameters, including the intensity of a given peak (also sometime referred to as a band), or ratios of two given peaks.

For the field of Proterozoic palaeobiology, the peaks of interest are often associated with carbon. In perfectly crystalline graphite a single first order peak occurs at 1582 cm^{-1} , attributed to stretching of the C-C bonds in basal graphite planes (known as the ‘G’ or ‘graphite’ peak) (Jehlicka *et al.* 2003). Second order peaks occur at $\sim 2695\text{ cm}^{-1}$ and 2735 cm^{-1} . Imperfectly crystallised graphitic carbons including kerogens have additional peaks at $\sim 1355\text{ cm}^{-1}$ (known as the ‘D1’ or ‘disordered’ peak) and $\sim 1620\text{ cm}^{-1}$ (‘D2’; occurring as a shoulder to the ‘G’ peak), and a single broad second order peak around 2700 cm^{-1} . The specific position, width and relative intensities of these peaks vary somewhat depending on the degree of ordering of the carbon, and these parameters have been characterised in carbon of varying metamorphic grade in an attempt to use Raman as an indicator of the antiquity of carbon in ancient rocks (Tice *et al.* 2004). This is by no means an exact science, since the starting composition of organic material in different metamorphic terrains, both geographically and temporally, may differ. Putative carbonaceous microfossils should, however, exhibit very similar Raman spectral features as other carbonaceous material in the same rock specimen because both should have undergone the same maturation processes. Raman spectra cannot be used to unequivocally determine the biogenicity of an ancient carbonaceous object because similar spectra to those of biogenic kerogens are seen in laboratory synthesised abiological disordered carbonaceous material (Pasteris & Wopenka 2003). However, the co-occurrence of kerogenous composition with features that optically

resemble cellular material provides promising preliminary data regarding biogenicity that can be further tested using higher spatial resolution techniques (see below).

As with CLSM, the highest quality data is obtained from specimens close to the surface of a thin section, and it has been suggested that for viable 3D maps of kerogen to be produced, the entire specimen of interest should be no more than 6-8 μm below the surface (Marshall & Olcott Marshall 2013). The best data will come from specimens lying under translucent minerals such as quartz, $\sim 1\text{-}5\text{ }\mu\text{m}$ below the surface of a thin section; microfossils associated with phases that fluoresce strongly under the laser excitation beam may not provide usable spectra. Care must also be taken not to confuse the carbon signature of interest with that produced by: (i) the polymer used to attach the thin section to the glass slide; (ii) any coating that may have been applied to the section during previous analyses; (iii) overlapping peaks in the vicinity of carbon peaks – of particular note here is the 1320 cm^{-1} peak of hematite (Marshall *et al.* 2011). The carbon spectrum can also be artificially modified by using too high a laser power, or by analyzing right at the surface of a thin section that has been polished (Fries & Steele 2011). Both of these should always be avoided. Raman can also be used to elucidate some structural information from the minerals that host putative microfossils. Several minerals produce Raman spectral peaks that vary in intensity depending on their crystallographic orientation relative to the incoming laser. This feature can be used, for example, to image the distribution of quartz crystallographic axes to see whether putative microfossil material occurs between grain boundaries, is enclosed by entire grains, or occurs in cracks (Fries & Steele 2011).

Examples of Raman applied to Proterozoic microfossils include a study by Fries and Steele (2011) who mapped the carbon D/carbon G peak intensity ratio (an indicator of graphite

domain size) to show micron-sized variation in the structure of kerogen within and around examples of *Huroniospora* from the 1878 Ma Gunflint Formation. This potentially reflects initial heterogeneities in the biological material. Also within the Gunflint Formation, Wacey *et al.* (2013) used Raman to demonstrate that *Gunflintia* microfossils were dominantly carbonaceous in composition, but were preserved as pyrite in microenvironments where anoxia had permitted pyrite formation via the metabolic activity of sulfate reducing bacteria. Raman has been used extensively by Schopf and colleagues to characterize Proterozoic microfossils (Schopf *et al.* 2005, 2008; Schopf & Kudryavtsev 2005, 2009), culminating in the Raman Index of Preservation (RIP). This correlates the geochemical maturity of the kerogen, the fidelity of microfossil preservation, H/C and N/C ratios of organic material, and the metamorphic grade of the rocks, and includes examples from 22 chert units ranging in age from 400 Ma to 2100 Ma (Schopf *et al.* 2005).

Micro-Fourier Transform Infrared Spectroscopy (micro-FTIR)

Micro-FTIR is a vibrational spectroscopy technique that provides complementary information to that obtained from organic material using Raman. In particular, it provides data pertaining to the functional groups attached to carbon chains and their bonding environment within organic material (Mayo *et al.* 2004; Dutta *et al.* 2013; Chen *et al.* 2015). Different peaks in an IR spectrum arise due to different vibrational behaviour in the bonds of groups such as CH₂, CH₃, C-N, C=O and others. FTIR can be applied non-destructively but requires doubly polished thin sections, and the main drawback is currently the limited spatial resolution obtainable, with recent studies reporting only a ~15 µm² spot size in transmission mode (Qu *et al.* 2015). This is sufficient to characterize larger Proterozoic acritarchs in palynological extracts (Aroui *et al.* 1999; Marshall *et al.* 2005) and groups of smaller filamentous and coccoid microfossils (Igisu *et al.* 2009), but is insufficient to determine the

difference between, for example, wall chemistry and internal chemistry of most Proterozoic organisms. The spatial resolution problem may be circumvented somewhat by using micro-FTIR attached to a synchrotron beamline, where spot sizes of $< 5\mu\text{m}$ have been achieved for some parts of the spectra (Bamberg 2016). However, this may require more specialist, often extremely difficult, sample preparation (e.g. $<20\mu\text{m}$ thickness, unglued slice).

Of particular interest are data from extant microorganisms suggesting that FTIR may provide 'domain specific' information, whereby specific components (e.g., lipids) of different domains of life (i.e., prokaryote, eukaryote, and archaea) may possess characteristic ratios of CH_2 and CH_3 groups in their IR spectra (Igisu *et al.* 2009, 2012). This has led to FTIR being used in Proterozoic assemblages in an attempt to decode the phylogenetic affinity of microfossils (Igisu *et al.* 2009, 2014). The study of Igisu *et al.* (2009) analysed microfossils in their mineral matrix and thus concentrated on the CH_x ($2500\text{--}3100\text{ cm}^{-1}$) region of the spectrum. This type of research is very much in its infancy and a better understanding, both of the changes in CH_2/CH_3 during postmortem alteration processes, and of the spectral parameters of differentiated cells in multicellular organisms, is required in order for these data to become a robust domain level signature. Insufficient data currently exist for comparisons of organic material from different terranes and of different metamorphic grades using this technique. Nevertheless, FTIR analyses from the 850 Ma Bitter Springs Formation, Australia, and 1878 Ma Gunflint Formation, Canada, suggest that organisms in these fossil assemblages belong to Bacteria rather than Archaea or Eukarya (Igisu *et al.* 2009). Likewise, combined FTIR and Raman data from the 1485 Ma Wumishan Formation, China (Qu *et al.* 2015), suggests that the organic material here is derived from prokaryote cyanobacteria, and is characterised by a rather homogenous and low CH_3/CH_2 . Finally, FTIR data from acritarchs from the ~ 575 Ma Tanana Formation, Australia, suggest that *Tanarium* are likely

eukaryotic micro-algae but *Leiosphaeridia* may be Bacteria (Igisu *et al.* 2009, based on data presented in Marshall *et al.* 2005).

Destructive high spatial resolution techniques

Focused Ion Beam Milling and Scanning Electron Microscopy (FIB-SEM)

SEM has traditionally been of limited use in characterizing Proterozoic microfossils in geological thin sections since the majority of microfossils are embedded within the thin section, and below the reach of this surface-based technique. SEM has, however, provided high spatial resolution morphological data from the surfaces of individual microfossils in acid etched rocks, or those extracted from their host rock using acid maceration. This has revealed, for example, delicate wall ultrastructure that could not be resolved under the light microscope (Javaux *et al.* 2004; Moczydlowska & Willman 2009; Agic *et al.* 2015).

Recently, the use of SEM in Precambrian palaeobiology has been reinvigorated by a new generation of dual beam instrument, where the user has access to both a focused ion beam (FIB) and an electron beam (see Young & Moore, 2005 for overview). Thus, a highly focused beam of heavy ions (usually Ga⁺) can be used to sputter ions from the sample surface, essentially cutting into the sample with very high (nano-scale) precision (see Wirth 2009 for details). The electron beam can be used to image the results. Additional detectors can be inserted to image backscattered electrons (BSE) as well as secondary ones, permit elemental analysis (using an energy dispersive X-ray spectroscopy (EDS) detector), or even phase detection and crystallographic mapping (using an electron backscatter diffraction (EBSD) detector). FIB milling can be used to cut into or through specific features in a thin section or rock chip, allowing the structure perpendicular to the surface to be better visualized (Westall *et al.* 2006). Furthermore, a number of sequential slices can be milled through an

object, with images or other data acquired after each slice is milled. The latter is termed FIB-SEM nano-tomography and permits the 3D reconstruction and visualisation of microfossils at very high spatial resolution (see Wacey *et al.* 2012 for details). The resolution attainable is essentially dictated by the size of the object to be analysed in 3D, plus the available time, although instrumental resolution limits may come into play for very small objects. Slice thicknesses are set by the user and can be smaller than 50 nm, however, for practical reasons 100-200 nm slices have commonly been used. Proterozoic microfossils have been visualized using FIB-SEM nano-tomography from the 1878 Ma Gunflint Formation (Wacey *et al.* 2012, 2013), ~1700 Ma Ruyang Group (Schiffbauer & Xiao 2009; Pang *et al.* 2013) and ~1000 Ma Torridon Group (see below). In the former, FIB-SEM data were key in revealing heterotrophic bacteria attached to, and fossilized in the act of decomposing, larger organisms (Wacey *et al.* 2013). Drawbacks of FIB-SEM nano-tomography include its destructive nature – the analysed specimen is completely consumed and only a digital record of its existence will remain – plus the restrictive timescales involved in both analyzing objects larger than about 30 µm in diameter (24 hours or more beam-time required), and in processing and reconstructing the data. A number of options exist for processing and visualizing such data (and data from other 3D techniques such as X-ray CT), ranging from freeware products such as *SPIERS* (serial palaeontological image editing and rendering system; Sutton *et al.* 2012), *Drishti* (Limaye 2012), and *Blender* (Garwood & Dunlop 2014) to more advanced (but expensive) products such as *AVIZO* (www.vsg3d.com). The choice of software will depend on budget, time constraints, quality of the raw data, and whether one is interested in producing just images, or images plus movies (see Sutton *et al.* 2014 for an overview of the options).

Transmission Electron Microscopy (TEM)

TEM covers a number of separate sub-techniques that can all be performed in a transmission electron microscope. Most simply, TEM is a very high spatial resolution imaging technique, capable of resolving objects separated by as little as ~0.1 nm. A standard TEM image results from variable electron scattering as a beam of electrons is accelerated at high voltage through an ultrathin (ideally ≤ 100 nm) sample; a true high-resolution image (HRTEM) is a phase contrast image with atomic-scale resolution, allowing the visualisation of the arrangement of atoms within a sample (Williams & Carter 2009). This provides information about the crystallinity of a sample, its lattice structure and any defects it may have.

Sample preparation is key to obtaining high quality data, and in this regard FIB has revolutionized the use of TEM in Precambrian palaeobiology. Before the advent of FIB, sample preparation for TEM involved either grinding up a rock, extracting organic material by acid maceration, or using ion polishing, meaning that the context of the putative microfossils was often lost, it was very difficult to obtain samples of uniform (and ultrathin) thickness, and contamination was widespread. FIB-milling now allows individual microfossils, or even specific parts of individual microfossils to be targeted with great accuracy in their host thin section, then ultrathin wafers (typically about $15\text{ }\mu\text{m} \times 10\text{ }\mu\text{m} \times 100\text{ nm}$) can be extracted from below the surface of the thin section (hence eliminating the possibility of contamination) and mounted on a TEM grid (see Wacey *et al.* 2012 for an overview).

In addition to morphology, a number of other parameters can also be analysed on many TEMs, including elemental composition, bonding and oxidation state, crystal structure (leading to mineral identification), and crystal orientation. The elemental composition of a sample can be determined at the nano-scale using either energy-dispersive X-ray

spectroscopy (EDS) or by isolating and mapping specific energy windows from an electron energy loss spectroscopy (EELS) spectrum. The fine structure of peaks within an EELS spectrum can also be used to shed light on the bonding and oxidation state of the element of interest, for example distinguishing disordered carbon from graphite (Buseck *et al.* 1988), and Fe²⁺ from Fe³⁺ (Calvert *et al.* 2005). For advanced crystallography and mineral identification, selected area electron diffraction (SAED) in the TEM provides quantitative information on the distances between atomic planes in crystalline materials, and permits the orientation of several grains of the same mineral to be compared to one another.

In Proterozoic palaeobiology, TEM has been used for several decades with early images of microfossils extracted from their host rock in the ~850 Ma Bitter Springs Formation, Australia, shown in Oehler (1977). A number of studies have investigated the wall architecture of Proterozoic acritarchs in an attempt to decode their taxonomic affinities, because TEM can detect variations in electron density and texture of different layers within cell walls at nm-scale resolution. These include studies from: the ~575 Ma Tanana Formation, Australia (Arouri *et al.* 1999; Moczydlowska & Willman 2009) where recognition of a trilaminar sheath structure was part of a suite of evidence suggesting the microfossils were chlorophyte algae; the 650 Ma Chichkan Formation, Kazakhstan (Kempe *et al.* 2005) where TEM helped to elucidate the nanostructure of carbon particles making up the cell wall; and the ~1450 Ma Roper and Ruyang Groups of Australia and China respectively (Javaux *et al.* 2004) where at least four different types of wall ultrastructure suggested a greater diversity of eukaryote clades in these deposits than could have been recognised by standard optical techniques. TEM has also been used to investigate the interplay of microfossil walls with the minerals in which they have been preserved, with studies from the 1878 Ma Gunflint Formation showing how nano-grains of silica disrupt the carbonaceous walls of bacteria as

they are fossilized (Moreau & Sharp 2004; Wacey *et al.* 2012), and data from the ~750 Ma Draken Formation, Svalbard, showing both the cell membrane and cytoplasm of the coccoid microfossil *Myxococcoides* embedded within nano-grains of silica (Foucher & Westall 2013). Finally, TEM data from the ~580 Ma Doushantuo Formation, China, helped to decode the relationships between preserved microfossils and the phosphate granules in which they are contained and suggested that phosphate precipitation was likely to have been microbially mediated (She *et al.* 2013).

Secondary Ion Mass Spectrometry (SIMS)

SIMS as applied to the field of Proterozoic palaeobiology is a surface analysis technique, whereby the elemental or isotopic composition of a sample can be determined at moderate to high spatial resolution and with great sensitivity (i.e. many elements can be detected even when present only at the parts-per-billion level). The surface of a sample is sputtered with an ion beam and the secondary ions ejected from the sample are collected and analysed using a mass spectrometer (see Ireland 1995 for details). Two different types of SIMS instruments are commonly used in in palaeobiological investigations:

- 1) The large radius SIMS, used to accurately determine the stable isotope ratios of key biogenic elements (e.g., carbon, sulfur), plus ratios of radiogenic isotopes in order to date rock formations containing microfossils (see for example Stern *et al.* 2009; Williford *et al.* 2013; Farquhar *et al.* 2013). Such instruments can analyse objects as small as ~10-20 μm diameter and the isotopic data can have a precision better than 0.5 parts per thousand (‰).
- 2) The NanoSIMS, which has a different geometry and is thus capable of element (ion) mapping with a lateral resolution down to ~50 nm (see Kilburn & Wacey 2015 for details). The NanoSIMS can also make accurate isotopic measurements from objects smaller than 5 μm , albeit with poorer precision (generally >1 ‰) than the large radius SIMS.

Both forms of SIMS can be applied to surface features in standard geological thin sections and rock chips, although some specialist sample preparation is needed in order that the sample and appropriate standards can be correctly mounted together within the instrument. This generally involves mounting pieces of thin sections or rock chip alongside analytical standards in resin discs. SIMS is partially destructive in that layers of surface material (as deep as ~200 nm during isotope analysis with large radius SIMS) are consumed during the analysis. Small specimens may be entirely consumed by the analysis, whereas larger specimens can be repolished post analysis to look like new!

A number of Proterozoic microfossils have been analysed by SIMS in the last 15 years. House *et al.* (2000) were the first to determine the carbon isotope composition of individual microfossils using material from the ~850 Ma Bitter Springs and 1878 Ma Gunflint Formations, finding $\delta^{13}\text{C}$ signatures (-21 to -45 ‰) consistent within specific metabolic pathways (namely the Calvin Cycle and acetyl-CoA). This work was recently refined by Williford *et al.* (2013) who analysed microfossils from four Proterozoic assemblages (Gunflint, Bitter Springs, plus ~650 Ma Chichkan Formation and ~740 Ma Min'yar Formation) with greater precision and reproducibility. They were able to show considerable variability of $\delta^{13}\text{C}$ within individual assemblages that may reflect the preservation of original metabolic differences between different components of each biota, and also potential heterogeneities in molecular preservation in single microfossils. It must be noted at this stage that non-biological reactions are able to produce similar $\delta^{13}\text{C}$ fractionations (McCollom & Seewald 2006), so a $\delta^{13}\text{C}$ value must be supported by a definitive biological morphology in order to prove the biogenicity of ancient carbonaceous objects.

SIMS has also been used to investigate metabolic pathways involving sulfur in Proterozoic organisms. Wacey *et al.* (2013) determined the $\delta^{34}\text{S}$ composition of pyritised microfossils from the 1878 Ma Gunflint Formation, finding sulfur fractionations ($\delta^{34}\text{S} = +7$ to $+22$ ‰) consistent with pyrite formation via the activity of sulfate reducing bacteria in sulfate starved sediment porewaters. In the same study, Wacey *et al.* (2013) used NanoSIMS to map the residual carbon and nitrogen associated with the pyritised microfossils and found reproducible differences in the preservation of organic material between two different types of organism (*Huroniospora* versus *Gunflintia*). *Gunflintia* was poorly preserved which suggests that it was more prone to decay by heterotrophic bacteria (that also mediated pyrite formation) than *Huroniospora*. NanoSIMS mapping of organic microfossils in the ~850 Ma Bitter Springs Formation has shown the co-occurrence of carbon, nitrogen and sulfur in such microstructures (Oehler *et al.* 2006) and attempts have been made to quantify the ratios of nitrogen and carbon (N/C) to distinguish different components of microbial communities, or to distinguish biology from co-occurring abiotic organic material (Oehler *et al.* 2009; Thomen *et al.* 2014) although the SIMS community has yet to agree upon the robustness of these methods.

A PROTEROZOIC CASE STUDY: THE 1200-1000 MA TORRIDONIAN LAKES

The effectiveness of combining multiple high-spatial-resolution, *in-situ* techniques is demonstrated here using a case study of microfossils from the 1200-1000 Ma Torridonian Supergroup of Northwest Scotland. Not all techniques described above were applied to the Torridonian material in order to avoid duplication of data and in order to keep costs and processing time to reasonable levels. For example, we felt in this case that higher quality 3D morphological data could be acquired using FIB-SEM rather than CLSM, and that detailed chemistry could be better (and more cheaply) determined using TEM rather than X-ray

spectroscopy. Below we present data obtained from light microscopy, SEM, μ CT, laser Raman, NanoSIMS, TEM and FIB-SEM nano-tomography which together provide a detailed characterisation of a number of components of the Torridonian biota.

Methods

Optical microscopy

Polished and uncovered petrographic thin sections of 30 μ m and 100 μ m thickness were examined under *Nikon Optiphot-Pol* and *Nikon Optiphot-2* microscopes with 4x, 10x, 20x, 40x and 100x (oil immersion) lenses at the Department of Earth Sciences, University of Oxford, and with a *Leica DM2500M* microscope with 4x, 10x, 20x and 50x lenses at the Centre for Microscopy Characterisation and Analysis (CMCA), The University of Western Australia. Images were captured using *Synchroscopy* imaging software (*Acquis* and *Auto-montage*) at Oxford, and using *Toupview* imaging software at CMCA. Post processing, for example colouring of cells in Figures 2 and 3, was carried out in *Adobe Photoshop* (*GIMP* is an open source alternative).

SEM analysis of palynological specimens

Palynological samples were prepared at the Department of Animal and Plant Sciences, University of Sheffield, using conventional acid maceration techniques (Grey 1999). Following HCl-HF-HCl acid maceration, the residues were sieved using a 10 μ m mesh. They were then treated to a heavy liquid separation using zinc chloride, followed by further sieving at 10 μ m. The organic residues were mounted directly onto glass slides using epoxy resin. SEM imaging was carried out using a *JEOL JSM-840A* SEM located at the Department of Earth Sciences, University of Oxford.

604 *X-ray μ CT*

605 CT scans were performed at the Manchester X-ray Imaging Facility using: a *Nikon Metris*
606 225/320 kV X-ray CT system in a customized bay (tungsten reflection target; current/voltage
607 of 130 μ A/80kV; no filtration; 3142 projections of 708 ms exposure collected with a 2000 \times
608 2000 detector; reconstructed dataset 5.1 μ m voxels); and a *Zeiss Xradia Versa 520* (standard
609 transmission target; current/voltage of 62 μ A/160kV; standard in-built, high energy 2 Zeiss
610 filter; 4x optical magnification, 501–1001 projections of exposures between 0.5 and 2
611 seconds, collected with 4x binning using a 2000 x 2000 detector; reconstructed datasets with
612 1–2 μ m voxel size). Additional propagation-based phase-contrast scans were performed at the
613 TOMCAT beamline of the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland;
614 1001 projections of 700ms exposure; 37 KeV monochromatic beam; 4x objective; a LAG:Ce
615 100 μ m scintillator; reconstructions based on both attenuation and phase used to create
616 datasets with 1.625 μ m voxels). Datasets were reconstructed using the *SPIERS* software suite
617 (Sutton *et al.* 2012) following the methods of Garwood *et al.* (2012), and *Drishti* (Limaye
618 2012) following the methods of Streng *et al.* (in press).

619

620 *Laser Raman*

621 Laser Raman analyses were carried out at the University of Bergen using a *Horiba LabRAM*
622 *HR800* integrated confocal Raman system and *LabSpec5* acquisition and analysis software.
623 Samples were standard uncovered geological thin sections allowing optical and chemical
624 maps to be superimposed. All analyses were carried out using a 514.5 nm laser, 100 μ m
625 confocal hole, 1800 grating and 50x objective lens. The laser was focused at least 1 μ m
626 below the surface of the thin sections to avoid surface polishing effects. For mineral
627 identification from Raman spectra, dual acquisitions were taken from each analysis point,

each with an acquisition time of 4 s. Raman maps were acquired with a 1.5 μm spatial resolution.

TEM of FIB-milled wafers

TEM wafers were prepared using two dual-beam FIB system (*FEI Nova NanoLab*) at the Electron Microscopy Unit (EMU) of the University of New South Wales (UNSW), and Adelaide Microscopy at the University of Adelaide. Electron beam imaging was used to identify microfossils of interest in standard polished thin sections coated with c. 30 nm of gold, allowing site-specific TEM samples to be prepared. The TEM sections were prepared by a series of steps involving different Ga^+ ion beam energies and currents (see Wacey *et al.* 2012), resulting in ultrathin wafers of c. 100 nm thickness. These TEM wafers were either attached to *Omniprobe* copper TEM holders or deposited on continuous-carbon copper TEM grids. TEM data were obtained using a *FEI Titan G2 80-200* TEM/STEM with *ChemiSTEM Technology* operating at 200 kV, plus a *JEOL 2100* LaB_6 TEM operating at 200 kV equipped with a *Gatan Orius* CCD camera and *Tridiem* energy filter. Both instruments are located in CMCA.

NanoSIMS

Ion mapping was performed using a CAMECA NanoSIMS 50 at CMCA, with instrument parameters optimized as described in Wacey *et al.* (2011). Analysis areas were between 12 x 12 μm and 25 x 25 μm with a resolution of 256 x 256 pixels (so each pixel measures between 47 nm and 98 nm), with a dwell time of 5-15 ms per pixel, and a primary beam current of ~ 2.5 pA. Secondary ions mapped were $^{24}\text{C}_2^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{28}\text{Si}^-$, $^{32}\text{S}^-$, and $^{56}\text{Fe}^{16}\text{O}^-$, and charge compensation was achieved by using the electron flood gun.

FIB-SEM nano-tomography

Sequential FIB milling and SEM imaging was carried out on a *Zeiss Auriga Crossbeam* instrument at the Electron Microscopy Unit of UNSW, using the method of Wacey *et al.* (2012, 2014). Key parameters were adjusted to suit the specific size and nature of each sample of interest. In summary: initial trenches were milled using a 9 nA beam current; the imaged face was cleaned using a 2 nA beam current; ion beam current for slice milling was 2 nA; electron beam voltage for imaging varied between about 800 V and 5 kV; step sizes between slices were between 75 nm and 200 nm; and image capture times were around 30 seconds per frame. In some cases, dedicated trenches were milled in order to obtain elemental (EDS) maps of microfossils that were not subsequently milled for 3D analysis.

In order to visualize the data, FIB-SEM images were stacked, aligned and cropped using *SPIERSalign* (Sutton *et al.* 2012). The resultant stacks were imported into *SPIERSedit* (Sutton *et al.* 2012) where a number of masks were added to segment individual components (e.g., cell walls, cell contents) of the microfossil assemblage. The resulting files were exported and loaded into *SPIERSview* (Sutton *et al.* 2012) to generate the 3D surface renderings.

Results

Critically, before the Torridonian microfossils were subjected to the high-spatial-resolution, *in situ* microanalysis described here, multiple seasons of fieldwork had been completed in order to gain a firm understanding of the geological context of the host rocks. In addition, over 100 thin sections and hand samples had been studied in order to understand the depositional context and post-depositional history of the rocks, and to isolate only the very best and most promising samples for further study. A large amount of optical microscopy

work had also been completed in order to form an estimate of the morphological diversity of the biota. This work has all been peer reviewed and published (Strother *et al.* 2011; Callow *et al.* 2011; Battison & Brasier 2012; Strother & Wellman, 2015) thus giving a firm platform on which to build with this high-resolution work. A summary of some of the most common components of the Torridonian biota as observed by optical microscopy is given in Figure 1 for reference.

SEM data

As may be expected, the range of morphologies visible in SEM analysis (Fig. 2) is broadly comparable to those observed within thin sections of the phosphate (Fig. 1, plus Battison & Brasier 2012). Many simple vesicles and tubular morphotypes are observed, with SEM imaging affording enhanced resolution of their shape and wall structure. In particular, differences in the physical responses of structures to compression hint at differences in cell wall architecture. Two principal wall responses are observed. Thicker walled (wall at least 1 μm thick) specimens accommodate flattening with broad rounded velvet-like folds, or large creases (Fig. 2a). In contrast, thin-walled vesicles ($<0.5 \mu\text{m}$) accommodate compression with fine wrinkles irregularly distributed across the surface, and are apparently more prone to small tears (Fig. 2b). The flattening of these walls during preparation does not permit resolution of any ultrastructural lamination, but synthesis of taphonomic response and wall thickness may be used to enhance interpretation of microfossils studied by optical microscopy.

A number of unique forms of microfossils are also observed in SEM. This is likely due to the processing of larger quantities of material during preparation by acid maceration, as well as the enhanced resolution afforded by SEM imaging. Of note are two morphotypes, the first

(Fig. 2c) comprising a vesicle around 50 μm in diameter, ornamented with regular pits around 10 μm across, with each pit possessing a raised 'collar' approximately 2 μm wide and 2 μm high. This form bears some resemblance to the basal vesicle of *Cheilofilum hysteriopsis* Butterfield (see Butterfield, 2005 fig. 8 and fig. 10) or the freshwater green microalga *Botryococcus braunii* (see Vandenbroucke & Largeau 2007 plate e) in its possession of flanged openings. The second form (Fig. 2d) is a spherical hollow vesicle around 20 μm in diameter, with a spongy textured wall, and irregularly distributed, rounded or sub circular holes \sim 1-3 μm across. This morphotype is particularly notable for its retention of three-dimensional structure following maceration, indicating significant rigidity of the wall. In addition, distributed abundantly amongst the structurally distinguishable vesicles and sheaths, is non-vesicular membranous organic matter, with an irregularly pustulate and pitted texture, and an amorphous architecture. The size and nature of this material is likened to the amorphous extra-polymeric substances (EPS) secreted by mat-forming organisms in modern microbial ecosystems (cf. Paction *et al.* 2007), but could also be amorphous kerogen. This material is occasionally seen contained within thin sections as a light-walled membrane, but its texture and extent is clearer under SEM analysis.

Of particular note, amongst the vesicles, sheaths and putative EPS are small coccoid or bacillate forms seen to be colonizing, to varying degrees, some of the larger fossil structures. These are associated with pits within those larger structures, and are apparently embedded within a membrane that links them to the host fossil (Fig. 3). We interpret these forms as fossils of heterotrophic bacteria preserved feeding on the larger Torridonian microbial flora, and this interpretation reinforces observations made previously using light microscopy (see Battison and Brasier, 2012, fig.9, where evidence for heterotrophy includes roughly circular

holes in large microfossil vesicles and inferred clumps of heterotrophic bacteria
pseudomorphing decayed vesicles).

X-ray μ CT data

Microtomography was explored as a method to investigate the petrographic context of cellular material and was also tested in order to determine whether individual microfossils could be detected and their 3D morphology characterized. Scans of rock chips from the Cailleach Head Formation using a *Nikon Metris* 225/320 kV X-ray CT system with 5.1 μ m voxels revealed phosphate nodules as a slightly denser phase that could be distinguished from the surrounding matrix sediment (Fig 4a, purple). It also suggested that phosphate was present in small quantities close to, but exterior to the main nodule. Rounded concentrations of a very dense phase, most likely to be an iron rich mineral such as pyrite or iron oxide, were shown to be present both within and outside the nodule (Fig. 4a, gold). Hence, CT could be employed in future investigations as a pre-screen of rock fragments in order to determine the best position within the rock to cut thin sections. Within the phosphate nodules, the *Nikon* CT scans detected phases of lower density that may be organic microfossils. However, the spatial resolution of this instrument was insufficient to determine if these lower density objects were indeed microfossils or simply lower density sediment grains (e.g. quartz) scattered through the phosphate nodules. Higher resolution scans of a different rock chip (with 1.625 μ m voxels) conducted at the Swiss Light Source demonstrate a complex sedimentary texture - here both phosphate and other dense phases are present in the form of evenly spaced rounded- to angular- fragments within the scanned rock chips (Fig. 4b), with no evidence of well-formed nodules of phosphate. The lack of evidence for nodules suggest that this rock chip would not be a promising target for further investigation of microfossils.

CT scans of a sub-portion of the sample examined in the *Nikon* instrument, performed using a *Zeiss Xradia Versa 520* with voxels of c. 1.5 μm detected a small number of low density objects that strongly resemble microfossils observed in thin sections (Fig. 4c-d, f-g). These objects are analogous to some of the largest and darkest-walled vesicles seen in thin sections (Fig. 4e, h) and CT permits their viewing from multiple orientations in 3D space. These putative fossils are also frequently found close to the very high density phases (presumably iron oxide or pyrite). The combined evidence suggests that μCT at this resolution is only capable of detecting the largest and thickest-walled components of the Torridonian biota. We also suggest that the increased density contrast when such fossils occur in close proximity to iron oxide or pyrite aids detection by CT. The remaining components of the biota (e.g. examples shown in Fig. 1) are essentially invisible on X-ray CT scans conducted at these resolutions. The biggest challenge for future work will be identifying workflows to isolate known microfossils for future scanning.

Raman data

Raman data inform upon the dominant mineralogy of the Torridonian microfossils and their surrounding matrix, plus the structure and thermal history of any organic carbon present. Raman maps from the Cailleach Head Formation (Fig. 5a-c) demonstrate that the microfossils are indeed carbonaceous (Fig. 5b) and that the dominant fossilizing phase is apatite (Fig. 5c). Raman also shows that intracellular inclusions (Fig. 5a arrows), common in many of the spheroidal fossils from this formation, are also carbonaceous in composition. Hence, these inclusions likely represent plasmolysed (shrunken) cell contents or, in some cases, could represent a fossilised cell nucleus. Raman spectrum in the first order region of carbon, show the two main bands (**D1** at about 1350 cm^{-1} and G at about 1600 cm^{-1}) characteristic of disordered carbonaceous material. The **D1** band is very broad (full width at

half peak maximum (FWHM) of $\sim 120 \text{ cm}^{-1}$ with a shoulder at its low wavenumber side. This shoulder is caused by a small band at $\sim 1150 \text{ cm}^{-1}$ which is only observed in very disordered carbonaceous material (Marshall *et al.* 2005). The G band appears to have been shifted considerably from its value in crystalline graphite (1582 cm^{-1}) to a value of $\sim 1610 \text{ cm}^{-1}$. This reflects an overlap of the G band with a well-developed disorder band (D2) at $\sim 1620 \text{ cm}^{-1}$. The spectrum indicates that the carbonaceous material has very weak structural organization, has experienced little or no metamorphism (cf. Wopenka & Pasteris 1993), and is consistent with previously suggested maximum heating of only $\sim 100^\circ \text{C}$ (Stewart & Parker 1979).

Not all microfossils are preserved purely as carbon. In the Stoer Group, Raman reveals that significant portions of microfossil walls have been pyritised, although some carbonaceous signal remains (Fig. 5d-f). The matrix mineralogy is also different here, with typical phases including calcite and albitic feldspar (Fig. 5g-h). These data indicate that different suites of lakes within the Torridonian had different chemistries, with those of the Stoer Group being sulphate-rich and phosphate-poor compared to those of the Cailleach Head and Diabaig Formations (see Parnell *et al.* 2016, this volume, for further details on contrasting fossil preservation in these lakes).

NanoSIMS data

NanoSIMS was used as an additional tool to determine whether the microfossils were composed of carbonaceous material and then to determine if any additional elements of biological interest were preserved within their cell walls or intracellular space. NanoSIMS uniquely revealed significant (but not quantifiable) amounts of nitrogen and sulfur within cellular material from the Diabaig Formation (Fig. 6). These data were collected from FIB-

milled wafers and so the nitrogen and sulfur come from cell walls located below the surface of a thin section; this negates the possibility that these biological signals come from surface contamination and provides an improvement on previous NanoSIMS methodology where such ion mapping was performed on surface features (e.g. Oehler *et al.* 2006, 2009). The co-occurrence of C, N and S in microstructures that have cellular morphology is strong evidence of the biogenicity of such structures; while this is less relevant to the Torridonian material whose biogenicity is well accepted, it is a very useful tool for the investigation of older and/or more controversial fossil material. Building up a database of the C, N and S concentrations of different types of organic material may also be useful in the future to help determine if different components of cells (i.e. wall, membrane, nucleus, cytoplasm) can be preserved in exceptional circumstances. NanoSIMS also revealed the nature of some non-carbonaceous intracellular inclusions within the Diabaig Formation; in optical microscopy these inclusions are ruby red in colour (Fig. 6a), and NanoSIMS shows that they are iron oxides (Fig. 6e-f) and at least some occur in direct contact with the inner cell wall. These inclusions are rare, found in <1% of Torridonian microfossils, but may indicate a unique intracellular chemistry in this small proportion of specimens.

TEM data

TEM data reveal the chemistry of the fossilizing mineral phases and the ultrastructure of the microfossils at a spatial scale (nm) unattainable by any other technique. For example, ChemiSTEM (**STEM-EDS**) elemental mapping combined with selected area electron diffraction has shown that phosphate is not necessarily the dominant mineral responsible for exceptional microfossil preservation in the Cailleach Head and Diabaig Formations (cf. Raman and optical data). In fact, the minerals immediately adjacent to most vesicle walls are Fe-rich clay minerals of the chlorite group or K-rich clay minerals approximating illite (Fig.

7; see Wacey *et al.* 2014 for details on clay mineral identification); phosphate only dominates at some distance (tens to hundreds of nm) away from the cellular material. The interior of many microfossils are also filled with K-rich clay minerals (Fig. 7), although phosphate grains are also common in many cell interiors (e.g., Wacey *et al.* 2014, fig. 8). STEM-EDS in the TEM detects small C and F peaks in the phosphate spectra confirming that the phosphate is the common low temperature form often associated with fossils, francolite (carbonate fluorapatite).

TEM imaging reveals sub-components of microfossil walls that were not previously recognized. In many cases a presumed single, thick vesicle wall is shown to comprise multiple components. These can include a thicker inner wall sitting within a thinner outer wall, perhaps suggesting a cyst housed within a vegetative cell, or even more complex arrangements of up to four distinct layers within a ‘wall zone’ (Fig. 7). Such arrangements are too complex for simple prokaryote cells. Hence, this strongly suggests a eukaryotic component to the biota. These complex layered walls are also preserved in clay minerals. Hence, the combined data suggest that the fidelity of microfossil preservation may be enhanced by early precipitation of clay minerals, and that microfossil preservation in clay minerals may be of even higher quality than in phosphate.

FIB-SEM data

Two types of data were acquired using FIB-SEM, chemical and 3D morphological. Chemical data were acquired by simply slicing into a microfossil using a FIB and then analyzing the chemistry of a cross section through the microfossil using SEM-EDS. This provided similar data to STEM-EDS in the TEM but at a more flexible spatial scale (i.e. could be applied to larger fossils, albeit at lower spatial resolution). These data reinforced those acquired using

TEM, showing that in fossils with complex walls (interpreted as eukaryotes) clay minerals occurred in direct contact with microfossil walls, in between multiple walls, and in microfossil interiors, while calcium phosphate tended to occur exterior to the fossil (Fig. 8). In simpler prokaryote fossils, the pattern was less defined, with phosphate mixed with clay minerals typically occurring both exterior and interior to the cell (Fig. 9a-b).

Morphological data in three dimensions were acquired using FIB-SEM nano-tomography whereby sequential FIB slicing was followed by imaging using the SEM (see methods above). This provided an excellent visualization of cellular material located below the surface of the thin section (Fig. 9b) that would otherwise have been hidden by overlying fossil material (Fig. 9a). In addition, individual cells and cell contents could be visualized from multiple orientations in 3D space (Fig. 9c-f). This is particularly useful for accurately locating the position of organic intracellular inclusions (Fig. 9c-f). In the example presented here these inclusions are most likely shrunken remnants of the cytoplasm of simple prokaryote cells, but in future it may be possible to detect the remnants of eukaryotic nuclei or organelles, if preserved using such methods.

CONCLUSIONS

Here we have provided an overview of the types of high-resolution techniques currently available to those interested in characterizing Proterozoic microfossils and their associated minerals and fabrics. Techniques have been classified either as non-destructive, hence applicable to all material including holotypes, or destructive, hence applicable in cases where conservation of the specimen is not a requirement. Non-destructive techniques include laser Raman, CLSM, SEM, infrared spectroscopy, X-ray CT and X-ray spectroscopy, although to obtain the highest spatial resolution data using the latter two methods, specialized (and partly

destructive) sample preparation is required. Destructive techniques include SIMS where the surface layers of a microfossil are sputtered away during analysis, TEM where an ultrathin slice must be extracted from the microfossil, and FIB-SEM nano-tomography which consumes the entire specimen during analysis.

Maximum information is gained by the consilience of multiple approaches to a microfossil assemblage, but in reality there will be some tradeoff between time and budget constraints, efforts to conserve the best specimens, and the spatial resolution required. The destructive techniques of TEM and FIB-SEM provide the greatest spatial resolution while SIMS uniquely provides isotopic data. A sensible workflow would involve analysis of petrographic context and a significant number of representative specimens using non-destructive avenues, followed by focused analysis of fewer specimens by destructive means.

A case study from the Torridonian of northwest Scotland, a microfossil assemblage whose importance has recently been highlighted by work led by Martin Brasier, demonstrated the additional insights that these high resolution techniques can offer. Microtomography provided a rapid way to determine the locality of phosphate nodules that house microfossils, and other petrological details. SEM revealed a number of new morphotypes not previously recognized in optical work and hinted at different taphonomic responses by different types of cell and vesicle walls. TEM revealed the fine scale distribution of mineral phases in and around cellular material, and showed that clay minerals played an important part in the exceptional preservation of this biota. Raman together with NanoSIMS revealed details of the organic material making up the cells, including its thermal maturity and biochemistry in terms of C, N and S content. Finally, FIB-SEM nano-tomography provided a detailed 3D

view of a number of fossilized cells, including the location of the remains of organic cell contents.

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FIGURE CAPTIONS

Fig 1. Optical microscopy of Torridonian microfossils, demonstrating common morphotypes present in the assemblage. **(a)** Highly degraded dark-walled vesicle. **(b)** Pristine dark-walled vesicle. **(c)** Light-walled vesicle, potentially possessing a double wall. **(d)** Cluster of light-walled spheroidal unicells, most with a dark spot indicating potential preservation of cell contents. **(e)** Cluster of light-walled cells with mutually adpressed cell walls. **(f)** Pair of spheroidal unicells with very prominent dark inner sphere. **(g)** Partially decomposed filamentous sheath. **(h)** Filamentous sheath with bulbous termination housing potential spheroidal cell. **(i)** Colony of light-walled elliptical cells comparable to *Eohalotheca lacustrina* described by Strother & Wellman (2015). **(j)** Pair of cells that may have divided shortly before fossilisation, each containing a dark spot. Scale bars are 20 µm for **(a-i)** and 10 µm for **(j)**.

Fig. 2. Torridonian microfossils imaged and analysed by SEM-SE, coloured for easier interpretation. **(a)** Large, thick-walled vesicle, showing 'velvet-like' folds. **(b)** Smaller, thin-walled vesicles with a crinkled surface and finely irregular outline. **(c)** Vesicle with large hemispherical pits bounded by raised rims or 'collars'. **(d)** Subspherical rigid vesicle retaining a 3D structure, and bearing many irregular rounded holes. Pink coccoid structures attached to the vesicles in **(a-b)** are potential fossil heterotrophs (see also Fig. 3). Sample CAI-7, macerated from phosphate from Cailleach Head. All scale bars are 10 µm.

Fig. 3. Evidence of bacterial heterotrophy in SEM images. **(a-b)** Rounded pits and occasional holes, irregularly distributed on the surface of larger vesicles' walls, **(b)** is enlargement of boxed area in **(a)**. **(c-d)** Collapsed coccoid or bacillate cells ~5 µm across, occupying pits in

the walls of larger vesicles, occasionally with a thin raised lip, (d) is enlargement of boxed area in (c) with heterotrophs false coloured pink. (e) Densely packed colony of coccoid and bacillate cells (pink) continuous with amorphous degraded vesicular or EPS material (grey-green). (f) Higher magnification of colony in boxed area of (e), showing collapsed coccoid and bacillate structures arranged randomly with possible supporting and sheathing membrane. Sample CAI-7, macerated from phosphate from Cailleach Head. Scale bars are 20 μm for (a, c, e) and 10 μm for (b, d, f).

Fig. 4. X-ray microtomography analysis of Torridonian rock chips. (a) Reconstruction of a CT scan of a rock chip using the *Nikon* instrument (voxels c. 5 μm), highlighting part of a phosphate nodule (purple) within a quartz-rich sediment (grey), plus a number of higher density grains that are likely pyrite or iron oxide (gold). (b) Reconstruction of an X-ray scan of a second rock chip using the Swiss Light Source Synchrotron (voxels 1.625 μm). This shows a mixture of phosphate and other denser phases rather evenly distributed through the rock chip with no distinct phosphate nodule. (c-d and f-g) Reconstruction of two putative vesicles identified in a higher resolution CT scan using the *Zeiss Xradia Versa* instrument (voxels c. 1.5 μm). The light micrograph images (e and h) show specimens observed in thin sections that may be analogous to those identified using CT. Scale bar is 2 mm for (a) 500 μm for (b) and 20 μm for (c-h).

Fig. 5. Raman analysis of microfossils from the Torridonian Supergroup. (a) Optical photomicrograph of two coccoid microfossils from the Cailleach Head Formation, each containing dark interior spheroids (arrows). (b) Raman map of the carbon G $\sim 1600\text{ cm}^{-1}$ peak showing that the microfossils have carbonaceous walls and the dark interior spheroids are also carbonaceous. This suggests that they are clumps of degraded cellular material or

remnants of a cell nucleus. (c) Raman map of the major calcium phosphate (apatite) $\sim 960\text{ cm}^{-1}$ peak showing that a large proportion of the mineralising phase is apatite. The patchy appearance of the apatite suggests the presence of further mineral phases, interpreted to be clay minerals as detected in higher resolution SEM and TEM analyses (see Figs 7-8). (d) Optical photomicrograph of a microfossil from the Stoer Group. Raman maps of the carbon G $\sim 1600\text{ cm}^{-1}$ peak (e), pyrite $\sim 380\text{ cm}^{-1}$ peak (f), calcite $\sim 1090\text{ cm}^{-1}$ peak (g), and albite $\sim 510\text{ cm}^{-1}$ peak (h) demonstrating that the microfossil is partially pyritised but some carbonaceous composition remains, and that the sediment is dominantly calcite and feldspar. Scale bars are $10\text{ }\mu\text{m}$.

Fig. 6. NanoSIMS analysis of a microfossil from the Diabaig Formation. (a) Optical photomicrograph of a light-walled spheroidal cell with ruby red intracellular particles. (b) Overview of a FIB-milled wafer prepared for NanoSIMS from the region indicated by the yellow line in (a). Note the contrast between the large dark-grey grains, which equate to the ruby red grains in (a), and the remainder of the wafer, plus holes in the wafer likely induced by excessive FIB-milling. (c) NanoSIMS ion map of nitrogen measured as CN^- . (d) NanoSIMS ion map of sulfur measured as S^- . (e) NanoSIMS ion map of iron oxide measured as FeO^- . (f) Three colour overlay of nitrogen (blue), iron oxide (red) and silicon (green) showing that the large dark grains are iron oxides and they are located just inside the cell wall (intracellular). The other mineral phases are dominantly clays and quartz. Scale bar is $20\text{ }\mu\text{m}$ in (a), and $5\text{ }\mu\text{m}$ for (b-f). Note scale bar in (c) also applies to (d-f).

Fig. 7. TEM analysis of a FIB milled wafer extracted from a Torridonian microfossil. (a) Optical photomicrograph of a dark-walled spheroidal microfossil from the Cailleach Head Formation. (b) Overview of the FIB-milled wafer extracted from the region marked by the

yellow line in (a) showing a complex wall structure and different mineral phases (indicated by different levels of grey within the image) inside and outside of the microfossil (from Wacey *et al.* 2014). (c) Three colour overlay of ChemiSTEM elemental maps of carbon (blue), aluminium (orange) and calcium (pink) from the region indicated by the dashed box in (b). Carbon represents the organic material of the microfossil walls, and at least four separate walls (or wall layers) can be seen. Calcium represents apatite, the dominant mineral phase outside of the microfossil. Aluminium represents clay minerals that infill the microfossil, occur between the walls of the microfossil and occur in minor amounts outside of the microfossil. Black areas are holes in the TEM wafer. Scale bar is 10 μm in (a), 2 μm in (b), and 1 μm in (c).

Fig. 8. FIB-SEM-EDS of a microfossil from the Cailleach Head Formation. (a) Optical photomicrograph of a dark walled spheroidal vesicle showing the location of the FIB-milled area and direction of view for the other panels in the figure (from Wacey *et al.* 2014). (b) Secondary electron image showing the FIB-milled face below the surface of the thin section. Shown below are energy-dispersive X-ray (EDS) elemental maps of the FIB-milled face shown in (b). Carbon (light blue) represents the organic microfossil walls, highlighting a thick inner cyst wall and thinner outer vegetative cell wall. Phosphorus (red), calcium (pink) and moderate levels of oxygen (green) represent apatite, the dominant fossilising mineral outside of the microfossil. Iron (blue), plus moderate amounts of silicon (turquoise), aluminium (orange) and oxygen represents Fe-rich clay, occurring between the two microfossil walls, replacing parts of the outer wall, and continuing for 1–2 μm outside the outer wall. Potassium (yellow), plus silicon, aluminium and oxygen represents K-rich clay restricted to the interior of the vesicle. Scale bars are 5 μm .

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1424 **Fig. 9.** 3D FIB-SEM nano-tomography of a Torridon microfossil. **(a)** Optical
1425 photomicrograph of a cluster of light-walled spheroidal cells from the Cailleach Head
1426 Formation (from Wacey 2014). **(b)** Example of a FIB-milled slice through the cluster of
1427 microfossils in the region indicated by the dashed line in **(a)**. Note that portions of at least 8
1428 cells can be seen in this image, some of which are hidden from view below other cells in the
1429 optical photomicrograph. Note also dark material inside the upper central cell (dashed arrow)
1430 **(c-f)** 3D model of the cell indicated by the solid arrow in **(b)** viewed from four different
1431 orientations, showing the location of preserved cell contents (blue) with respect to the cell
1432 wall (yellow). Note that in (f) part of the cell wall has been removed to better visualize the
1433 cell contents. Scale bar is 10 μm in **(a)**, and 5 μm for **(b-f)**. Note scale bar in **(c)** also applies
1434 to **(d-f)**.

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